REMARKS

Claims 7-32 are pending in the application as shown in the paper filed July 12, 2004. Claims 30-32 have been withdrawn as being drawn to non-elected species. Claims 7-29 are under active consideration.

Objection to the Drawings

Fig. 12 is objected to on the grounds that the figure and corresponding amendments to the specification allegedly "introduced new matter" (Final Office Action, pages 2-3. In particular, the Final Office Action alleges that the Domenighini reference was not properly incorporated into the instant disclosure by reference because "[m]ere reference to another application, patent, or publication is not an incorporation of anything therein into the application containing such reference for the purpose of the disclosure required by 35 U.S.C. 112, first paragraph" (Final Office Action, page 3). The Final Office Action further alleges that "the disclosure as filed addresses strains of LT in general (p. 5, lines 5-7) and does not reduce the genus of particular species addressed in Domenighini" (Final Office Action, page 3). The Final Office Action also alleges that "information on SEQ ID Nos 1-4 presented in the reference is essential material as it is being directly claimed in the amended claims" and "there is nothing in the specification as filed suggesting LT-A sequences having residues other than Ala at position 72, whereas SEQ ID Nos 3,4 on the figure present proteins with different residues (I and L) therein" (Final Office Action, page 3). For the reasons of record, Applicants reiterate their position that FIG. 12 does not add new matter.

Applicants respectfully point out that the Domenighini reference was properly incorporated by reference. See specification at page 10, lines 28-30, which states that all publications cited in the specification are incorporated by reference.

As set forth in M.P.E.P. § 608.01(p), Applicants must be afforded the opportunity to amend their specification to include material incorporated by reference and deemed "essential." See, also, In re Hawkins, 486 F.2d 569, 179 USPQ 157 (CCPA 1973); In re Hawkins, 486 F.2d 579, 179 USPQ 163 (CCPA 1973); In re Hawkins, 486 F.2d 577, 179 USPQ 167 (CCPA 1973). See, in particular, M.P.E.P. § 608.01(p), 2. Improper Incorporation ¶6.19 as follows:

> The incorporation of essential material in the specification by reference to a foreign application or patent, or to a publication is improper. Applicant is required to amend the disclosure to include the material incorporated by reference.

Thus, the amendment of the specification to include the material in FIG. 12 is proper and does not constitute new matter.

The Examiner mischaracterizes the context in which the reference of Domenighini is cited in the specification. Domenighini is clearly and unambiguously cited in the specification for **everything** it teaches in regard to LT-A sequences, including **all** the sequences of FIG. 12 (see, specification page 5, lines 25-31, emphasis added):

> It will be appreciated that in derivatives of LT-A, such as fragments, **or in LT-A proteins of different *E. coli* strains**, the amino acid residue to be mutated is that which corresponds to Ala-72 as defined for LT-A in Domenighini et al. [Molec. Microbiol. (1995) 15:1165-1167]. Ala-72 is located on the second turn of the alpha-helix in LT-A and faces the NAD binding site.

Thus, the specification cites Domenighini to show full-length sequences of LT-A proteins that may serve as reference sequences and the residue to be mutated that corresponds to Ala-72 in the reference sequences. Figure 12 corresponds to Figures 1 and 2 of Domenighini, showing an alignment of various wild-type LT sequences. Applicants further note that the sequences of Domenighini and those added to the specification were publically available at the time of filing.

In summary, Applicants reiterate that the incorporation of material deemed essential from the reference of Domenighini et al. is proper according to M.P.E.P. § 608.01(p). Therefore, **no** new matter was added by the previous submission of FIG. 12 or corresponding amendments, and withdrawal of the objection to the drawings is respectfully requested.

Objection to the Specification

The Specification is objected to under 35 U.S.C. § 132 on the grounds that the amendment filed 2/08/2005 allegedly introduced new matter in the disclosure. In particular, the Final Office Action alleges that "the listing of sequences SEQ ID Nos. 1-4 constitutes new matter for the same reasons as their description in the form of Fig. 12 – see the preceding objection to the Drawings" (Final Office Action, page 4).

Applicants respectfully disagree for the reasons discussed above regarding the objection to FIG.12. Applicants reiterate that the incorporation of material from the reference of Domenighini et al. was proper according to M.P.E.P. § 608.01(p). Therefore, **no** new matter was added by the previous submission of FIG. 12 or the corresponding amendments, and withdrawal of the objection to the specification is respectfully requested.

Written Description Rejection under 35 U.S.C. § 112, first paragraph

Claims 7-29 have been rejected under 35 U.S.C. § 112, first paragraph for alleged lack of an adequate written description. In particular, the Final Office Action alleges that "[t]he claims recite 8-meres of SEQ ID No. 1. The specification as filed addressed fragments of LT-A in general, but have not addressed fragments of particular size as now claimed" (Final Office Action, page 4). In addition, the Final Office Action alleges that "[t]he specification as filed addresses polynucleotides encoding full-length subunits of LT-A (p.7, lines 23-26) but does not describe polynucleotides encoding any other polypeptides comprising certain fragments of LT-A" (Final Office Action, page 5). The Final Office Action further alleges that "the DNA encoding protein, reads on DNA encoding full-length protein, but not on DNA encoding any other polypeptides comprising certain fragments of the protein" (Final Office Action, page 6). Applicants respectfully traverse the rejection.

The Examiner's attention is again directed to the specification at page 17, lines 10-19, where it is stated (emphasis added):

> A polypeptide or amino acid sequence "derived from" a designated nucleic acid sequence refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, **or a portion thereof** wherein the portion consists of at least 3-5 amino acids, and more preferably at least **8-10 amino acids**, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence. This terminology also includes a polypeptide expressed from a designated nucleic acid sequence.

Thus, the specification clearly describes polypeptide fragments of 8-10 amino acids. In addition, the specification states that "[r]eferences to LT-A also encompass fragments of LT-A provided that the fragment contains Ala-72" (see specification at page 5, lines 14-15), and it is clear that the invention also includes nucleic acids encoding such fragments. See specification, for example, at page 9, lines 15-20, where it is stated (emphasis added):

> According to a ninth aspect of the invention, there is provided a process for the production of DNA according to the fifth aspect of the invention comprising the steps of subjecting **a DNA encoding a wild-type LT-A or a fragment thereof** to site-directed mutagenesis.

In addition, Applicants direct the Examiner's attention to page 41, lines 35-38 of the specification, describing sources of wild-type LT-A-encoding sequences (*e.g.*, citing Pronk et al and Spicer et al.). Further, it is well within the purview of a skilled artisan to align sequences with SEQ ID NO:1; to determine which residue corresponds to Ala-72; and to substitute arginine for this residue. (See, *e.g.*, page 42 of the specification). Methods of including these polynucleotides in vectors, host cells and the like are similarly described in the specification and within the purview of the skilled artisan. (See, *e.g.*, pages 6-8 and 19 to 43 of the specification). It is also well within the purview of the skilled artisan, in view of the teachings of the specification, to construct fragments of LT-A containing residue 72 (numbered relative to SEQ ID NO:1) and to replace the wild-type residue with an arginine. (see, *e.g.*, page 5, lines 14-15; page 17, lines 10-19 and Examples of the specification). These fragments could readily be tested for toxicity (*e.g.*, in the well known Y1 cell assay). (see, *e.g.*, page 43. line 35 to page 44 line 21 for example of Y1 assay). Thus, the specification clearly conveys to a skilled artisan that applicants were in possession of the precisely claimed molecules at the time the application was filed. Accordingly, in light of Applicants' disclosure and state of the art at the time of filing, designing and using nucleic acid molecules encoding the claimed LT-A mutants and fragments thereof is well within the purview of a skilled artisan. Therefore, the claimed polynucleotides encoding LT-A mutants and fragments thereof are adequately described.

For at least the above reasons, Applicants respectfully request that the written description rejection under 35 U.S.C. § 112, first paragraph be withdrawn.

Enablement Rejection under 35 U.S.C. § 112, first paragraph

Claims 7-29 have been rejected under 35 U.S.C. § 112, first paragraph, on the grounds that the specification does not provide an enabling disclosure commensurate in scope with the claims. In particular, the Final Office Action alleges that "the instant application demonstrates that full length LT-A has reduced toxicity as compared to wild-type, (Figs 4,5), but does not demonstrate any octamers of LT-A that are detoxified compared to wild type LT-A" (Final

Office Action, page 6). The Final Office Action cites the reference of WO 93/13202 to allegedly show the lack of predictability in the art and to suggest that LT-A derivatives having the Arg72 substitution may remain toxic. In addition, the Final Office Action alleges that "there is no description in the claims or specification sufficiently identifying epitope sequence. Consequently, there is no guidance on what fragments are required to maintain immunogenecity [sic] of the fragments required by the claims" (Final Office Action, page 6). Applicants respectfully traverse the rejection for the reasons already made of record and on the following grounds.

The test of enablement is whether one of skill in the art could make and use the invention based on the specification as a whole. A specification must be taken as enabling in the absence of evidence to the contrary. The courts have consistently held that not every last detail of any invention need be described, "else patent specifications would turn into production specifications, which they were never intended to be." See, e.g., *In re Gay*, 309 F.2d 769, 774 135 USPQ 311, 316 (CCPA 1962) and *Staehelin v. Secher* 24 USPQ2d 1513, 1516 (BPAI 1992). Thus, the proper legal standard for determining enablement is whether the specification provides enough guidance as to the existence of methods and materials that allow one of skill in the art to practice the claimed invention using the disclosures in the specification coupled with information known in the art, without undue experimentation. (see, *e.g., In re Wands,* 8 USPQ2d at 1404, citing *In re Angstadt*, 190 USPQ 214 (CCPA 1976); and *Ex parte Forman*, 230 USPQ 546 (BPAI 1986)).

Furthermore, a patent application is presumptively enabled upon filing and it is incumbent upon the Patent Office to explain *why* it doubts the truth or accuracy of any statements in the disclosure. *In re Marzocchi*, 439 F.2d, 220, 223, 169 USPQ 367, 369 (CCPA 1971). Thus, Applicants request clarification regarding the Examiner's position, as well as support for the Examiner's assertion that Applicants' statements are not persuasive, either in the form of scientific literature, or by a declaration pursuant to 37 CFR §1.104(d)(2) which includes the Examiner's qualifications as one of skill in the art. Without such evidence, the rejection must fail.

The record is replete with evidence establishing that it would require, at the very most, routine experimentation for a skilled artisan to identify DNA sequences encoding the claimed fragments. In particular, the specification teaches how to design expression vectors that include DNA sequences encoding the claimed fragments (*see, e.g.,* pages 19-42). Claims 7-29 each require that the polypeptide encoded by the polynucleotide is an immunologically effective detoxified fragment of at least 8 amino acids in length and that this fragment contain a mutation in residue 72, as numbered relative to SEQ ID NO:1. As previously noted the specification further describes how these fragments of LT-A must include an arginine at residue 72, numbered relative to SEQ ID NO:1 (*see, e.g.,* page 5, lines 14-15). It is well within the purview of the skilled artisan, in view of the teachings of the specification, to construct fragments of LT-A containing residue 72 (numbered relative to SEQ ID NO:1) and to replace the wild-type residue with an arginine. (see, *e.g.,* page 5, lines 14-15; page 17, lines 10-19 and Examples of the specification). These fragments could readily be tested for toxicity for example, using the well known Y1 cell assay or the *in vivo* ileal loop assay described in the specification (see, *e.g.,* page 43, line 35 to page 44 line 21).

In other words, using standard molecular biological and immunological techniques well known to those working in the field, it would require only routine experimentation for a skilled artisan to follow the teachings of the specification and determine whether any DNA sequence encoding a fragment of LT-A as claimed was immunogenic and detoxified. Thus, the specification as filed more than amply satisfies the enablement requirement of Section 112, as one of skill in the art could make and use the claimed molecules without undue experimentation following the guidance set forth in the specification as filed.

With regard to the supposed unpredictability in the art regarding toxicity, Applicants have clearly shown that the Arg substitution for Ala-72 renders the LT-A protein detoxified and that the detoxified mutant retains immunogenicity (see specification, *e.g.,* at Examples 2 and 3). The Examiner has cited Applicants' own previous work as evidence of unpredictability regarding the ability of the Arg substitution to reduce toxicity. The earlier results allegedly showing that the LT-A72R mutant remains toxic (see, *e.g.,* Table I at page 46 of WO 93/13202) have been contradicted by work performed subsequently, both by Applicants and others. In the instant application, Applicants have presented new experimental data showing that the ADP-

ribosylation activity is reduced by two orders of magnitude in the LT-A72R mutant compared with the wild-type LT-A. According to Y1 cell assays, the LT-A72R mutant has 10^{-5} toxicity of wild-type LT-A (see specification, *e.g.*, at page 44, lines 17-18). In addition, the LT-A72R mutant exhibits 20-fold less toxicity than the wild-type LT-A in *in vivo* ileal loop assays (see specification, *e.g.*, at page 44, lines 19-20).

Furthermore, the inapplicability of WO 93/13202 to the pending claims is also evidenced by the enclosed reference of Beignon et al. (Infect. Immun. 70:3012-3019, 2002; Appendix A hereto), which further corroborates that the LT-A72R mutant exhibits reduced toxicity. In studies of mice immunized with the LT-A72R mutant, Beignon et al. show that the LT-A72R mutant is a potent immunogen with low toxicity that provides effective protection against challenge with LT.

Thus, there is a wealth of experimental data establishing that the A72R mutant has reduced toxicity, and the reliance on references supposedly showing that the mutation fails to reduce toxicity is unfounded. The specification teaches that a mutation of the recited residue (Ala-72) to Arg renders the protein detoxified and, accordingly, any fragment including the specified mutation would be similarly detoxified.

With regard to immunogenicity, it was well known by persons skilled in the art at the time of filing that the correlation between polypeptide structure (sequence) and immunogenic function is flexible. In other words, whereas essential residues are readily identifiable for enzymatic or toxic functions, any given polypeptide can tolerate multiple substitutions at various residues or deletions, while still retaining its immunogenic function. Thus, to the extent required, the correlation between structure and toxic/immunogenic functions is clearly laid out in the specification and claims.

Furthermore, the courts have emphatically rejected the notion that one of ordinary skill in the art must have reasonable assurance of obtaining positive results in all cases. *See, In re Angstadt,* 190 USPQ 214, 219 (CCPA 1976). So long as it is clear that some species render a method operative, the inclusion of some possible inoperative species does not invalidate the claim under paragraph 1 of 35 U.S.C. §112. *In re Cook*, 439 F.2d 730, 735, 169 USPQ 298, CCPA 1971; *Horton v. Stevens*, 7 USPQ2d 1245, 1247, Fed. Cir. 1988. Moreover, even evidence of the need for some experimentation does not invalidate a claim on ground of undue

experimentation, nor does it fulfill the PTO's burden of proof. (*In re Angstadt* at 218; *In re Morehouse*, 545 F.2d 162, 165, 192 USPQ 29, 32, CCPA 1976.)

Thus, in the pending case, Applicants are in no way required to show that each and every fragment of the LT-R72 mutant is immunogenic and/or detoxified. All that is required is that the specification teaches a skilled practitioner how to, without requiring undue experimentation, select a polypeptide including at least 8 residues and the Arg72 mutation, make a DNA sequence encoding this polypeptide, and test the polypeptide encoded by the sequence to determine if it is immunogenic and detoxified.

For the reasons of record and those reiterated above, the specification more than satisfies this requirement. The techniques needed to practice the claimed invention would be routine to the skilled artisan in light of the teachings of the specification. If, using the procedures set forth in the specification, the polypeptide encoded by the selected DNA sequence is not detoxified or is not immunogenic, it does not fall within the scope of the pending claims.

Furthermore, it is not necessary for Applicants to present working examples of "immunogenic" detoxified fragments. It is more than ample that the specification as filed teaches one of skill in the art how to make and use the fragments as claimed. As noted above, the fragments must include a mutation at a specific residue, which renders the polypeptide detoxified. With regard to immunogenicity, the specification clearly teaches how test the detoxified polypeptide (or fragment) for the requisite immunogenic activity. Any fragment that includes the detoxifying mutation but which is not immunogenic remains outside the scope of the claims. Moreover, in order to satisfy the enablement requirement, Applicants need only show that the experimentation required to test immunogenicity is routine.[1] For the reasons of record, Applicants have more than amply demonstrated that such testing is routine and, accordingly, the rejection is improper and should be withdrawn.

Simply put, the claimed fragments will be detoxified by virtue of the mutation at the specified residue, a finding set forth clearly in the specification as filed. Further, the immunogenic nature of any detoxified fragment can be readily tested and it is improper for the

[1] *See, also, United States v. Telectronics Inc.*, 8 USPQ2d 1217 (Fed. Cir. 1988), *cert. denied,* 490 U.S. 1046 (1989)), holding that routine experimentation, even if extensive (on the order of six or more months and tens of thousands of dollars), is not necessarily undue.

Office to require working examples to establish enablement.[2] Any "inoperative" embodiments are excluded from the scope of the claims and can be identified as such using routine experimentation.

For at least the above reasons, Applicant respectfully requests that the enablement rejection under 35 U.S.C. § 112, first paragraph be withdrawn.

Rejections under 35 U.S.C. §§ 102 and 103

Claims 7-29 remain rejected under 35 U.S.C. §§ 102 and 103 as being unpatentable over the reference of EP 145486. In particular, the Examiner alleges that the reference discloses sequences in which a fragment of an LT-A mutant has an Arg residue in place of an Ala. The Final Office Action further alleges that the claim limitations require only that any Ala in the sequence be replaced by an Arg on the grounds that "[a]ny Ala residue will be 'corresponding' to any other Ala residue because they are the same by their nature" (Final Office Action, page 8). Applicants respectfully disagree and traverse the rejection for reasons already made of record and reiterated herein.

The Examiner has again improperly construed the claims. The claims clearly require that the recited polynucleotides encode an LT-A fragment wherein the amino acid residue corresponding to Ala-72 of SEQ ID NO:1 is an arginine residue. There is no basis for the Examiner's assertion that the position of a residue, namely 72 of SEQ ID NO:1, is an irrelevant claim limitation.

The position of a residue in a structure determines the surrounding residues, proximity to the active site, how the structure is perturbed by introduction of a longer and positively charged side chain. Notably, Ala-72 is located on the second turn of the alpha-helix in LT-A and faces the NAD binding site (see specification, for example, at page 5, lines 29-31). An Arg substitution at this position dramatically lowers ADP ribosylation activity (see specification, for example, at page 43, lines 29-30). An Arg substitution elsewhere in the LT-A protein would likely have a different effect. Position in a protein is obviously relevant.

[2] *See, also*, references previously submitted (*e.g.*, Habeeb, Stylos), which provide explicit evidence that analysis of peptide fragments for their immunogenicity was routine at the time of filing.

When the claim is properly read as a whole, it is clear that the claims require the replacement of a residue at the position "corresponding to" Ala-72 of SEQ ID NO:1 with an arginine residue. The claims (and specification) specify the particular position of the residue that must be replaced, with respect to a reference sequence (SEQ ID NO:1). It is improper to ignore this explicit limitation of position and assert that any toxic protein that includes any Ala-Arg substitution reads on the pending claims.

Accordingly, to fall within the scope of the claims of the instant application, EP 145486 would have to disclose fragments including SLRSAHL**R** or **R**GQSILSG (where the bolded R indicates the replaced residue is at the position corresponding to residue 72 of SEQ ID NO:1. Such fragments are not disclosed in EP145486. In fact, in EP145486, the alanine residue corresponding to Ala-72 of SEQ ID NO:1 remains an alanine (see, residue 90 of the sequence cited on page 8 of the Final Office Action). Thus, the rejections under 35 U.S.C. §§ 102 and 103 are improper and should be withdrawn.

Rejection under 35 U.S.C. § 102(b)

Claims 7-29 remain rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Burnette et al. (U.S. Patent No. 5,770,203). In particular, the Examiner alleges that the reference discloses a cholera toxin comprising a fragment of residues 29-54 of the instantly claimed SEQ ID NO:1. The Final Office Action further alleges that "the referenced product satisfies the structural limitation of instant claim to contain an Arg residue" (Final Office Action, page 9). Applicants respectfully traverse the rejection.

Again, as discussed above, it is improper for the Examiner to ignore the position of the amino acid replacement. The claims recite the limitation that the polynucleotides encode an LT-A fragment wherein the amino acid residue corresponding to Ala-72 of SEQ ID NO:1 is an arginine residue. Contrary to the Examiner's assertions, the claim does not require an alanine residue to be replaced. Any residue corresponding to position 72 of SEQ ID NO:1 may be replaced with an Arg. However, the position of the residue must correspond to position 72 of SEQ ID NO:1. The Examiner has aligned the sequence of cholera toxin with SEQ ID NO:1 and found a fragment with sequence homology over the region of residues 29-54 of SEQ ID NO:1. Notably, this fragment does not include residue 72 of SEQ ID NO:1. Furthermore, in the

sequence disclosed by Burnette et al., a valine residue resides at the position corresponding to Ala-72 of SEQ ID NO:1 of the instant application. Therefore, Burnette et al. fail to satisfy the claim limitation requiring that the fragment comprise an Arg residue at the position corresponding to Ala-72 of SEQ ID NO:1.

For at least these reasons, Applicants respectfully request that the rejection under 35 U.S.C. § 102(b) be withdrawn.

CONCLUSION

In light of the above remarks, Applicant submits that the present application is fully in condition for allowance. Early notice to that effect is earnestly solicited.

If the Examiner contemplates other action, or if a telephone conference would expedite allowance of the claims, Applicant invites the Examiner to contact the undersigned.

The Commissioner is hereby authorized to charge any fees and credit any overpayment of fees which may be required under 37 C.F.R. §1.16, §1.17, or §1.21, to Deposit Account No. 18-1648.

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Enclosures:

1. Appendix A: Beignon et al. (2002) Infect. Immun. 70:3012-3019.

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The LTR72 Mutant of Heat-Labile Enterotoxin of *Escherichia coli* Enhances the Ability of Peptide Antigens To Elicit CD4$^+$ T Cells and Secrete Gamma Interferon after Coapplication onto Bare Skin

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Application of antigens with an adjuvant onto bare skin is a needle-free and pain-free immunization procedure that delivers antigens to the immunocompetent cells of the epidermis. We tested here the immunogenicity and adjuvanticity of two mutants of heat-labile enterotoxin (LT) of *Escherichia coli*, LTK63 and LTR72. Both mutants were shown to be immunogenic, inducing serum and mucosal antibody responses. The application of LTK63 and LTR72 to bare skin induced significant protection against intraperitoneal challenge with a lethal dose of LT. In addition, both LT mutants enhanced the capacity of peptides TT:830-843 and HA:307-319 (representing T-helper epitopes from tetanus toxin and influenza virus hemagglutinin, respectively) to elicit antigen-specific CD4$^+$ T cells after coapplication onto bare skin. However, only mutant LTR72 was capable of stimulating the secretion of high levels of gamma interferon. These findings demonstrate that successful skin immunization protocols require the selection of the right adjuvant in order to induce the appropriate type of antigen-specific immune responses in a selective and reliable way. Moreover, the use of adjuvants such the LTK63 and LTR72 mutants, with no or low residual toxicity, holds a lot of promise for the future application of vaccines to the bare skin of humans.

Recently, bare skin has emerged as a potential alternative route for vaccine delivery (11, 27). This is because the skin is rich in immunocompetent cells (4, 36), and when antigens are applied with a suitable adjuvant either in solution (1, 11, 13, 34) or with a patch (14), they induce potent immune responses. The development of noninvasive immunization procedures, which can be needle free and pain free, is a top priority for public health agencies. This is because many current immunization practices are unsafe, particularly in developing countries due to the widespread reuse of nonsterile syringes (27). Therefore, the topical application of vaccines is attractive since it has the potential to make vaccine delivery more equitable, safer, and efficient. Furthermore, it would greatly facilitate the successful implementation of worldwide mass vaccination campaigns against infectious diseases.

For the induction of an effective immune response, the antigen is normally coapplied onto hydrated bare skin with an ADP-ribosylating exotoxin as an adjuvant (i.e., *Vibrio cholerae* cholera toxin [CT] or *Escherichia coli* heat-labile enterotoxin [LT]) (1, 11, 13, 34). Both CT and LT are composed of five nontoxic B subunits held together in a pentamer (responsible for binding to the cell membrane), surrounding a single A subunit, which is responsible for toxicity. The A subunit consists of two distinct structural domains: the A1 domain, which displays the ADP-ribosyltransferase activity in the cytosol of the target cells and the A2 domain that interacts with the B-subunit (35). These toxins are responsible for the cause of a debilitating watery diarrhea (35). Moreover, they are potent immunogens and exert an adjuvant effect on antigens presented simultaneously at the mucosal surfaces (26). When CT and LT are applied to bare skin it appears that they are well tolerated even at a high dose without any apparent sign of local or systemic toxicity (14). However, it is obvious that there would be some serious concerns for their use in humans. This has prompted researchers to genetically detoxify these toxins while retaining their adjuvanticity. By site-directed mutagenesis, several mutants have been generated with significantly reduced ADP-ribosylating activity and toxicity compared to the holotoxin (5, 6, 20, 30, 37). Two of these mutants, LTK63, which is devoid of enzymatic activity and toxicity (containing a serine-to-lysine substitution in position 63 of the A subunit), and LTR72, which retains ca. 1% of the wild-type ADP-ribosylating activity and reduced toxicity (containing an alanine-to-arginine substitution in position 72 of the A subunit), have been extensively tested and shown to be good adjuvants after mucosal coadministration with protein and peptide antigens (24, 29). Furthermore, these mutants have been shown to be very useful tools for examining the role of ADP-ribosylation in immunomodulation (32).

Since the LTK63 and LTR72 mutants are promising candidates for human use (28), we hypothesized that their use might allow us to circumvent the potential hazards of LT and CT after topical application. Therefore, we sought to investigate their immunogenicity and to test their adjuvanticity to peptide antigens after their coapplication to bare skin. Both mutants were shown to be effective immunogens, conferring protection against challenge with LT and enhancing the capacity of coadministered peptides to induce antigen-specific CD4$^+$ T cells. In addition, the LTR72 mutant was shown to stimulate the secretion of high levels of gamma interferon (IFN-γ).

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MATERIALS AND METHODS

Synthetic peptides. The synthetic peptides TT:830-843 [QYIKANSKFIGITE (C)] and HA:307-319 [PKYVKQNTLKLAT(C)] representing promiscuous (non-major histocompatibility complex-restricted) T-helper epitopes from tetanus toxin (7) and influenza virus hemagglutinin (25), respectively, were synthesized by using Fmoc (9-fluorenylmethoxy carbonyl) chemistry. The influenza virus NP:55-69 (RLIQNSLTIERMVLS) peptide representing a T-helper epitope from nucleoprotein was synthesized by using the same chemistry and was tested as a control peptide. After cleavage, the peptides were purified by preparative high-performance liquid chromatography and then characterized by analytical high-performance liquid chromatography and mass spectroscopy.

Mice. Female BALB/c mice, 6 to 8 weeks old at the start of the experiments, were purchased from Harlan (Gannat, France) and were maintained in the animal facility of the Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France.

Immunizations. Prior to immunization, mice were shaved on a restricted area of the abdomen (over an ca. 1- to 2-cm^2 surface area). During the immunization procedure the mice were under deep anesthesia after subcutaneous injection of 100 μl of solution of ketamine (Imalgene 1000 [15%]; Merial, Lyon, France) with xylasine (2% Rompun [9%]; Bayer AG, Leverkusen, Germany) for ca. 1 h to prevent grooming. Groups of BALB/c mice were immunized onto bare skin with a 30-μl volume of antigen solution (i) as a solution of 100 μg of TT:830-843 peptide with 50 μg of LT (Sigma) (eight mice), (ii) as a solution of 100 μg of TT:830-843 peptide with 50 μg of LTK63 (six mice), (iii) as a solution of 100 μg of TT:830-843 peptide with 50 μg of LTR72 (six mice), or (iv) as a solution of 100 μg of TT:830-843 peptide given in saline (two mice). At 2 weeks after priming, the mice were boosted by the same route with the same dose and formulation of antigen. In a separate experiment, the adjuvanticity of 50 μg of each of LT mutants was tested after coapplication with 100 μg of HA:307-319 peptide onto bare skin of BALB/c mice (two mice/group). Control mice were immunized with a mixture of 50 μg of CT and 100 μg of synthetic oligodeoxynucleotide (ODN) containing the CpG motif 1668 (5′-TCC ATG ACG TTC CTG ATG CT-3′) (18), purchased from MWG Biotech, Ebersberg, Germany. A booster application was given 14 days postpriming. No erythema was observed after the shaving or during and after the immunization procedure.

LT challenge. Groups of BALB/c mice were immunized onto bare skin with 50 μg of LTR72 (10 mice) or LTK63 (5 mice) mutant on days 0 and 14. Three weeks after the boost, immune mice and 11 nonimmune mice were challenged intraperitoneally with 50 μg (a 2.5 50% lethal dose) of recombinant LT in sterile saline (200 μl/mouse). After challenge, mice were monitored daily for morbidity and mortality.

Collection of vagina washes. Vagina washes were collected by gentle pipetting of 30 μl of sterile saline containing 0.5% bovine serum albumin (BSA) into and out the vagina lumen several times, followed by collection of the effluent. To limit the effect of estrous cycle on local antibody responses, vagina washes from two consecutive days were collected, pooled, centrifuged to remove particulate matter, and stored at −20°C until testing.

Measurement of antibody responses. Antibodies were measured by an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtiter plates (Falcon, Oxnard, Calif.) were coated overnight with 5 μg of LT/ml in 0.05 M carbonate-bicarbonate buffer (pH 9.6) at 37°C. The plates were blocked with 1% BSA in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) at 37°C for 2 h. After the plates were washed with PBS-T, serial twofold dilutions of serum or vaginal washes in PBS-T containing 0.25% BSA were made across the plate (final volume, 50 μl), and the plates were incubated at 37°C for 1 h. At the end of the incubation period, the plates were washed with PBS-T and incubated with 50 μl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; 1/20,000; Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.), anti-mouse IgA (1/5,000), or the anti-mouse IgG subclasses IgG1 and IgG2a (1/10,000 for IgG1 and IgG2a; Nordic Immunology, Tilburg, The Netherlands)/well that was Fc specific for 1 h at 37°C. Unbound conjugate was removed by washing the mixtures with PBS-T, and the enzymatic activity was determined as previously described (1). Data are expressed as antibody titers (log_{10}) corresponding to the reciprocal dilution giving an absorbance of 0.2 at 450 nm. Levels of total IgE antibodies in serum (serum samples were tested at a 1/20 dilution) were measured by a double-sandwich-based ELISA kit (OptEIA Set, mouse IgE; PharMingen, San Diego, Calif.) according to the manufacturer's instructions.

Assay for proliferative T-cell responses. Spleens were aseptically removed, and a single cell suspension was prepared in RPMI 1640 medium (Life Technologies, Cergy-Pontoise, France) supplemented with 100 IU of gentamicin/ml, 2 mM L-glutamine, 25 mM HEPES, and 1% heat-inactivated autologous mouse serum 2 weeks after the booster application of antigen formulation onto bare skin. A total of 4×10^5 viable splenocytes was cultured in 0.2-ml volumes in the presence of various concentrations of antigens. Supernatants collected after 72 h of culture were tested for their ability to support the proliferation of the interleukin-2 (IL-2)-dependent cell line CTLL-2 (9) after the culture of 10^5 cells/ml for 31 h. Then, 1 μCi of [^{3}H]thymidine was added 7 h before the end of the culture, and incorporation was measured with a Matrix 9600 Direct Beta counter (Packard, Downers Grove, Ill.). A standard curve performed with known concentrations of recombinant mouse IL-2 (0 to 45 U/ml; PharMingen) was used as an internal control to calculate the concentration of secreted IL-2.

Purification of CD4$^+$ T cells. CD4$^+$ T cells were separated from pooled splenocytes by using a magnetic cell separation device (MPC-6; Dynal, Oslo, Norway), Dynabead M-450 rat anti-mouse CD4$^+$ monoclonal antibody (Dynal), and DETACHaBEAD according to a well-established laboratory protocol (21) and the manufacturer's instructions. The purity of the positively selected CD4$^+$ T cells was assessed by flow cytometry with a rat anti-mouse CD4$^+$ monoclonal antibody (L3T4) (PharMingen) and the corresponding fluorescent monoclonal immunoglobulin isotype standard. Tested CD4$^+$ T cells were >90% pure (data not shown). Purified CD4$^+$ T cells (5×10^5 per well) were cultured in 96-well plates in the presence or absence of mitomycin-treated splenocytes as APCs (10^5 per well). For mitomycin treatment, 5×10^7 splenocytes (in 1 ml of PBS) were mixed with 100 μl of mitomycin C (Sigma; 500 μg/ml in PBS). After incubation at 37°C for 20 min, cells were washed three times in RPMI 1640 medium before use. Prior to coculture, APCs were incubated with different concentrations of peptide for 1 h at 37°C. Supernatants collected after 72 h of culture were tested for IL-2 secretion as described above.

IFN-γ ELISA assay. Levels of IFN-γ in culture supernatants collected after 72 h of culture of pooled splenocytes in the presence of antigen were measured by a double-sandwich ELISA with commercial antibodies from PharMingen. Briefly, polyvinyl Falcon plates were coated overnight at 4°C with 50 μl of 1 μg of purified rat anti-mouse IFN-γ (clone R4-6A2)/ml as the capture antibody in carbonate-bicarbonate buffer (pH 9.6). After being washed with PBS-T, the plates were blocked with 1% BSA and incubated at room temperature for 2 h. After the plates were washed with PBS-T, the supernatants were added in triplicate, and the plates were incubated at room temperature for 4 h. At the end of the incubation, the plates were washed, and 100 μl of a matched biotinylated rat anti-mouse IFN-γ (XMG1.2) monoclonal antibody (1 mg/ml) was added to each well. The plates were further incubated at room temperature for 1 h and then washed with PBS-T; avidin conjugated to peroxidase was then added to each well at room temperature for 30 min. The remaining steps of the assay were performed as described above (see the section on the measurement of antibody responses). The results are expressed as mean IFN-γ concentrations ± the standard deviation (SD), after extrapolation from a standard curve prepared with standard cytokine for each antigen concentration tested in duplicate.

Statistical analysis. Statistical analysis was performed by using the two-tailed Student's t test. Comparisons of survival rates after LT challenge were made by using the Kaplan-Meier product-limit method and analysis by the log rank test. A P value of ≤ 0.05 was considered to be statistically significant.

RESULTS

Immunogenicity of LT mutants after application onto bare skin. Figure 1a shows that both LT mutants elicited primary and secondary serum antibody responses. However, the mutant LTR72 was significantly more immunogenic than LTK63 after the boost ($P = 0.0002$). When antibody responses were compared to those induced by LT, the LTR72 mutant elicited significantly higher antibody titers ($P = 0.024$), whereas the LTK63 elicited significantly lower titers ($P = 0.005$) (Fig. 1a). The predominant IgG subclass after the boost was IgG1, with the ratios of IgG1 to IgG2a ranging from 2.02 for LT (IgG1 titer, 4.92 ± 0.12; IgG2a titer, 2.43 ± 0.46), 1.89 for LTK63 (IgG1 titer, 4.44 ± 0.07; IgG2a titer, 2.35 ± 0.61), and 2.5 for LTR72 (IgG1 titer, 4.54 ± 0.15; IgG2a titer, 1.81 ± 0.36). Both mutants induced detectable levels of IgG (average titers of 4.21 ± 0.17 and 2.61 ± 0.17 for LTR72 and LTK63, respectively) and IgA (average titers of 3.6 ± 0.16 and 2.4 ± 0.18 for LTR72 and LTK63, respectively) antibodies in vaginal washes, with

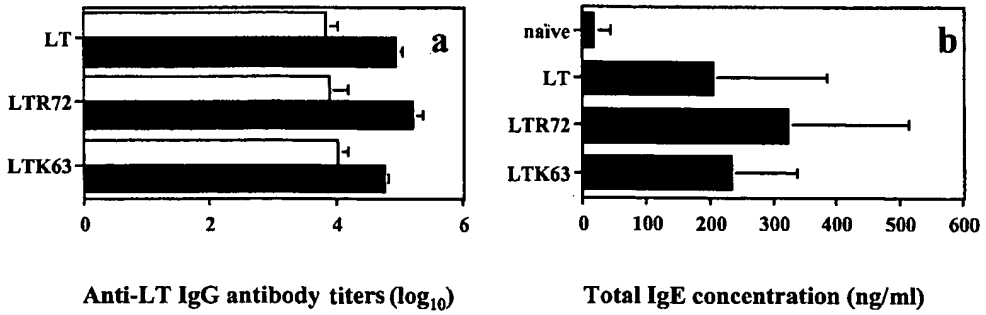

FIG. 1. Levels of anti-LT IgG (a) and total IgE antibodies (b) in serum. Mice were coimmunized onto bare skin with 100 μg of TT:830-843 peptide and 50 μg of LTK63 or with 50 μg of LTR72 mutant or 50 μg of LT as an adjuvant on days 0 and 14. Figure 1a presents the mean antibody titers ± the SD of groups of mice bled on days 14 (□) and 28 (■) after priming. Panel b presents the average concentrations of total IgE levels ± the SD in serum from groups of mice bled on day 28 after priming.

mutant LTR72 being more immunogenic. The total IgE levels in serum were significantly elevated compared to those of naive mice ($P = 0.0001$, $P = 0.0008$, and $P = 0.013$ for LTK63, LTR72, and LT, respectively) (Fig. 1b). However, no significant difference was observed between the two mutants ($P = 0.335$).

Protection against lethal LT challenge. To test the potential of skin immunization for the induction of protective immune responses, groups of BALB/c mice immunized with LT mutants onto bare skin were challenged via the intraperitoneal route with a lethal dose of LT. All immunized mice seroconverted prior to toxin challenge (mean antibody titers of 4.94 ± 0.19 and 4.58 ± 1.65 for LTR72 and LTK63, respectively, at 2 weeks before LT challenge). After intraperitoneal challenge with LT, immune mice were significantly protected ($P = 0.0001$ for LTR72 and $P = 0.004$ for LTK63) compared to the control nonimmune mice (Fig. 2).

Mutants LTK63 and LTR72 enhance the capacity of TT:830-843 peptide to elicit $CD4^+$ T cells. To determine whether the LT mutants can act as adjuvants, groups of mice were coimmunized with peptide TT:830-843 and LTK63 or LTR72 onto bare skin. Control mice were immunized by topically applying peptide TT:830-843 in saline or peptide TT:830-843 with LT. As shown in Fig. 3a, splenocyte cultures of mice immunized with peptide and LT mutants secreted significantly higher IL-2 levels upon in vitro restimulation with the homologous peptide compared to those of mice immunized with peptide alone ($P = 0.0001$). The levels of IL-2 secretion in culture supernatants of mice coimmunized with peptide TT:830-843 and LT were 22.5 ± 1.5, 17.85 ± 1.6, and 2.9 ± 1.75 U/ml in the presence of 5, 0.5, and 0.005 μg of homologous peptide/culture, respectively. Recall responses were also measured upon in vitro restimulation of splenocytes with tetanus toxoid (TTx) in groups of mice coimmunized topically with peptide and LT mutants but not in those immunized with the peptide in saline (Fig. 3b) or with LT (data not shown). These responses were dose dependent and significantly higher in splenocyte cultures of mice immunized with LTR72 ($P = 0.023$, $P = 0.0136$, and $P = 0.0007$ in the presence of 0.5, 0.05, and 0.005 μg of TTx/culture, respectively). No IL-2 secretion was measured upon in vitro restimulation with control peptide NP:55-69 (data not shown).

To assess the phenotype of proliferating T cells, $CD4^+$ cells were isolated with a magnetic cell separation device and cocultured with mitomycin-treated splenocytes pulsed with antigen. As shown in Fig. 3c, purified $CD4^+$ T cells of mice coimmunized with peptide TT:830-843, plus LTK63 or LTR72, secreted IL-2 in the presence of various concentrations of homologous peptide. Levels of IL-2 secretion in the culture supernatants of mice coimmunized with peptide TT:830-843 and LT were 24.65 ± 1.75 and 3.65 ± 0.9 U/ml in the presence of 5 and 0.5 μg of TT:830-843 peptide/culture, respectively. Recall proliferative responses were also measured after in vitro restimulation with various concentrations of TTx (Fig. 3d). After skin immunization, low levels of IL-2 secretion were also detected in the culture supernatants of inguinal lymph nodes restimulated with peptide but not with TTx (data not shown). Although peptide TT:830-843 induced proliferative T-cell responses, it did not elicit any detectable antibody responses (data not shown). This suggests that the peptide does not contain any B-cell epitope(s).

The secretion of IFN-γ is the hallmark feature of the Th1-type response that contributes to the clearance of viral infections and of other intracellular pathogens. Therefore, the IFN-γ response of immunized mice was measured in the supernatants of splenocyte cultures by ELISA. Mice immunized with TT:830-843 peptide plus LTR72 gave a strong IFN-γ

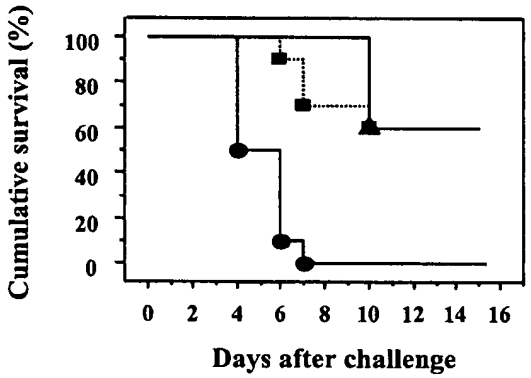

FIG. 2. Percent survival after intraperitoneal challenge with a lethal dose of recombinant LT (50 μg) of groups of BALB/c mice bare-skin immunized with LTR72 ($n = 10$, ■) or LTK63 ($n = 5$, ▲). Also shown are data for control nonimmune mice ($n = 11$, ●).

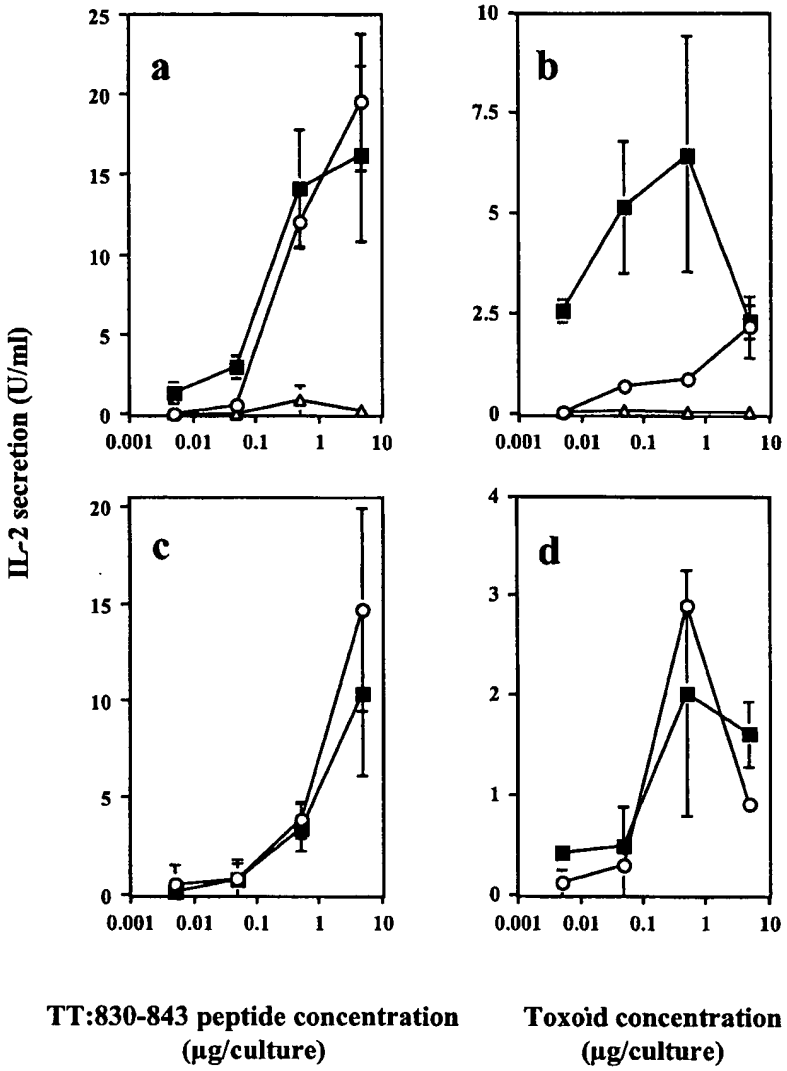

FIG. 3. Secretion of IL-2 by splenocytes (a and b) or purified $CD4^+$ splenocyte T cells (c and d) of mice coimmunized onto bare skin with 100 μg of TT:830-843 peptide plus 50 μg of LTK63 (○) or with 50 μg of LTR72 (■). Control mice were immunized with 100 μg of TT:830-843 peptide given alone (△). Splenocyte cultures or mitomycin-treated splenocyte APCs were restimulated in vitro or pulsed with various concentrations of TT:830-843 peptide (a and c) or TTx (b and d), and supernatants collected after 72 h of culture were tested for IL-2 secretion with the CTLL-2 IL-2-dependent cell line. Data are presented as IL-2 units/milliliter from hexaplicate cultures in the presence of peptide or TTx (triplicate cultures) ± the SD.

response in the presence of homologous peptide or TTx (Fig. 4). In contrast, IFN-γ was not detectable in the supernatants of splenocyte cultures of mice immunized with peptide in saline or with LTK63 mutant (Fig. 4). Splenocyte cultures of mice immunized with peptide TT:830-841 plus LT secreted IFN-γ in the presence of homologous peptide but not with TTx (Fig. 4). Restimulation of splenocyte cultures with control peptide NP: 55-69 did not produce any IFN-γ (data not shown).

LTK63 and LTR72 mutants enhance the capacity of HA: 307-319 peptide to induce proliferative T-cell responses. Since both LT mutants were shown to exert an adjuvant effect to the coadministered TT:830-843 peptide and mutant LTR72 in particular stimulated the secretion of high levels of IFN-γ, their adjuvanticity was retested with peptide HA:307-319 as an antigen. In addition, a group of mice were bare-skin immunized with peptide HA:307-319 plus a mixture of CT and an ODN containing the CpG motif 1668 as a positive control. This was based on the observation that the CT-ODN CpG mixture stimulates the production of high levels of IFN-γ (2). As shown in Fig. 5a, splenocyte cultures of mice coimmunized with peptide and mutant LTR72 secreted high levels of IL-2 upon in vitro restimulation with the homologous peptide. This response was significantly higher than that measured in the splenocyte cultures of mice coimmunized with peptide and LTK63 mutant ($P = 0.0001$ for all peptide concentrations tested). Splenocyte cultures of mice immunized with peptide plus LTR72 secreted IL-2 after restimulation with heat-inactivated influenza virus but not splenocyte cultures of mice immunized with peptide and LTK63 (Fig. 5b).

Peptide HA:307-319, like peptide TT:830-843, did not in-

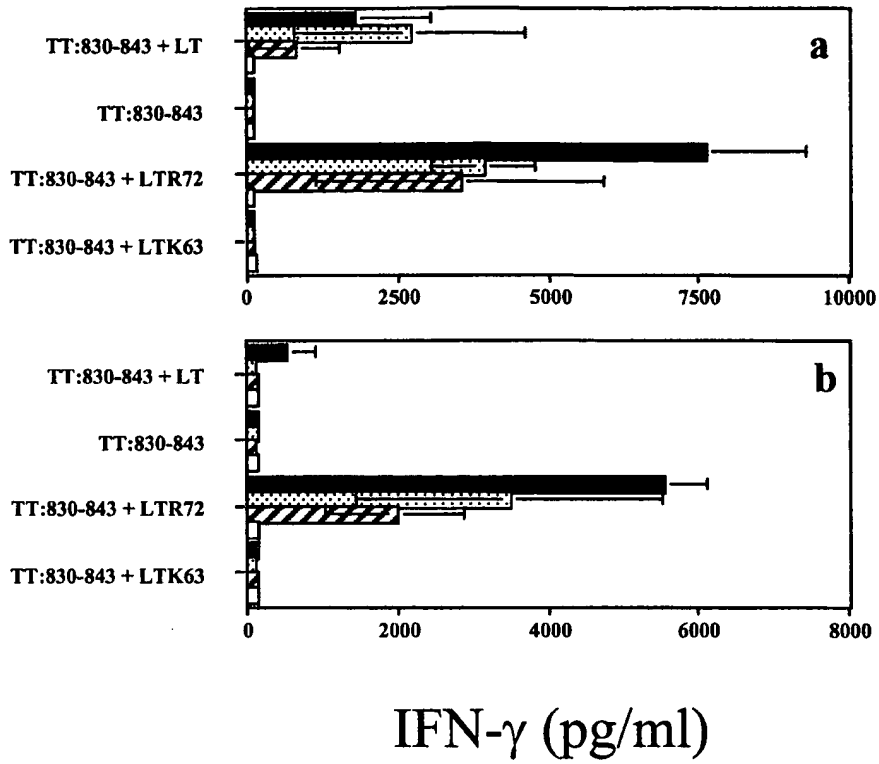

FIG. 4. Secretion of IFN-γ by TT:830-843 peptide-specific immune splenocytes after in vitro restimulation with various concentrations of the homologous peptide (a) or TTx (b) (■, 0.5 μg/culture; ▨, 0.05 μg/culture; and ▨, 0.005 μg/culture). Mice were coimmunized onto bare skin with 100 μg of TT:830-843 peptide and 50 μg of LTK63 or with 50 μg of LTR72 or 50 μg of LT as adjuvants. Control mice were immunized with 100 μg of TT:830-843 peptide given alone. After 72 h of culture, supernatants were collected and assayed for IFN-γ by ELISA. The findings with medium alone are also indicated (□).

duce any detectable levels of serum anti-peptide antibodies (data not shown), suggesting that it does not contain any B-cell epitope(s).

When the production of IFN-γ was measured in the supernatants of splenocyte cultures, only groups of mice that were coimmunized with peptide and LTR72 mutant or with the CT-CpG ODN 1668 mixture gave an IFN-γ response (Fig. 6).

DISCUSSION

In this study the immunogenicity and adjuvanticity of mutants of *E. coli* was tested after application onto bare skin. Both LTK63 and LTR72 mutants were shown to be immunogenic, inducing serum and secretory antibody responses. However, mutant LTR72 was the more potent immunogen. This finding extends previous observations on the immunogenicity of LT after skin application (1, 34), demonstrating that two of its mutants—LTK63, which is devoid of ADP-ribosylating activity, and LTR72, which is partially active—can be immunogenic. Furthermore, their ability to generate secretory antibody responses suggests that after application onto bare skin and after their diffusion through the hydrated stratum corneum (that disrupts its barrier function) they might influence the microenvironment of the epidermis. This in turn could favor the migration of antigen-pulsed Langerhans cells to lymphoid organs committed to initiating mucosal immune responses (8).

Adverse reactions to immunization are common and are normally tolerated for the benefit of immunity. In several instances high levels of IgE responses have been noticed with diphtheria toxoid vaccines (19). In the present study, total IgE levels were elevated in the sera of mice receiving LT, LTK63, or LTR72 as adjuvants. Although the exact role of the IgE responses remains to be elucidated, there is always the possibility that the presence of high levels of IgE might be associated with high risk of anaphylaxis, particularly in individuals with atopic predisposition (33). On the other hand, antigen-specific IgE appears to correlate with protection in diseases such as schistosomiasis (17).

Since mutants LTR72 and LTK63 were found to be highly immunogenic, the LT challenge mouse model was selected to evaluate whether immunization onto bare skin can induce protective immune responses against lethal systemic challenge with LT. Despite the vigorous dose of LT, immune mice were significantly protected. This finding is in agreement with observations demonstrating that skin immunization can generate protective immune responses against challenge with a lethal dose of toxins such as CT (12), LT (1), or tetanus (13).

The LTK63 and LTR72 mutants were also effective adjuvants since they enhanced the capacity of topically coapplied peptide antigens to induce antigen-specific $CD4^+$ T cells. Thus, LT mutants with low propensity for adverse side effects have adjuvant activity when they are topically applied, as has been demonstrated after their mucosal delivery (24, 29). Fur-

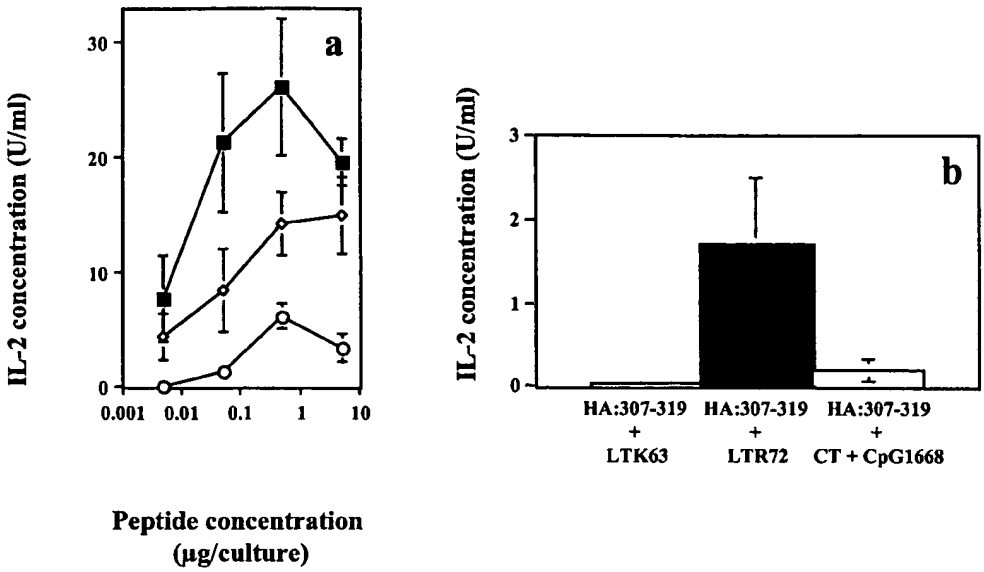

FIG. 5. Secretion of IL-2 by splenocytes of mice coimmunized onto bare skin with 100 μg of HA:307-319 peptide and 50 μg of LTK63 (○), with 50 μg of LTR72 (■), or with a mixture of 100 μg of CpG ODN 1668 and 50 μg of CT (◇). Splenocyte cultures were restimulated in vitro with various concentrations of peptide (a) or with 3×10^3 PFU of heat-inactivated influenza virus (b), and supernatants collected after 72 h of culture were tested for IL-2 secretion with the CTLL-2 IL-2-dependent cell line. The data are presented as IL-2 units/milliliter from hexaplicate cultures for the peptide (a) and from triplicate cultures for the virus (b) ± the SD.

thermore, their ability to enhance cellular immune responses to peptide antigens extends previous observations demonstrating their adjuvanticity to the topically coapplied diphtheria toxoid (34). However, it should be noted that in the present study two administrations instead of three and a lower dose of each mutant (50 μg instead of 100 μg) were tested.

The molecular mechanisms of adjuvanticity of these mutants are still unclear. From mucosal immunization studies it appears that the enzymatic activity is not an absolute prerequisite for adjuvanticity, since both LTK63 and LTR72 enhance immune responses to coadministered antigens (6, 30). The data of this report, along with the observations of Scharton-Kersten et al. (34), also support this view regarding skin immunization. Several adjuvants with no ADP-ribosylating activity, such as CpG motifs, lipopolysaccharide, muramyl dipeptide, alum, IL-2, and IL-12, have also been shown to enhance antibody responses to topically coapplied diphtheria toxoid (34). However, these responses were short lived and weaker than those induced by CT or LT (34). Thus, it appears that some ADP-ribosylating activity is necessary for enhanced adjuvanticity. The LTR72 mutant, which retains a residual enzymatic activity, is a more potent adjuvant compared to the nontoxic LTK63 mutant after intranasal (6, 10) or skin (34) immunization. Concerning the capacity of mutants to bind to cell surfaces via the G_{M1} gangliosides (35), studies with nonbinding mutants have demonstrated that binding was necessary for mucosal immunogenicity and adjuvanticity (15, 23). This is also supported by the recent findings of Beignon et al. (1) showing that preincubation of CT with G_{M1} gangliosides prior to its application onto bare skin results in a significant reduction of systemic and mucosal anti-CT antibody responses. In general, these mutants exert their adjuvant activity mainly on APCs to upregulate major histocompatibility complex and costimulatory molecules and to secrete cytokines (16, 38). This enables Langerhans cells to take up antigens, mature, and migrate to regional lymph nodes, where they present the antigen for the initiation of an adaptive immune response (16).

The ability of small molecules such as synthetic peptides to induce antigen-specific $CD4^+$ T cells after application onto bare skin is particularly important in the context of vaccine design and delivery, since $CD4^+$ T cells help B cells to produce antibodies that neutralize viruses and bacterial toxins, enhance the magnitude of cytotoxic T-cell responses to clear virus-infected cells, and regulate the immune responses to foreign antigens on the basis of cytokine profile they secrete (22). Moreover, the finding that mutant LTR72 preferentially stimulates an IFN-γ response could be advantageous, particularly for the clearance of intracellular pathogens (3). Recent reports have indicated that mutants LTK63 and LTR72 preferentially stimulate Th1- and Th2-type immune responses, respectively, when they are administered in small quantities via the intranasal route (31, 32). However, the type of antigen and the mode of intranasal delivery might influence the induction of a particular Th phenotype, since it has been shown in other systems with peptides (24) or parasite protein antigens (3) that only the LTR72 mutant induces an IFN-γ response. This is also consistent with the results obtained in this study. The exact mechanism(s) of preferential stimulation of IFN-γ secretion by the LTR72 mutant is not clear. If we take into account that ADP-ribosylating exotoxins and their mutants bind to immunocompetent cells (i.e., T cells, B cells, and APCs), it is very difficult to speculate the exact series of in vivo events that favor the secretion of IFN-γ by the LTR72 mutant. However, it could be argued that certain levels of cyclic AMP are critical for the signaling events that will eventually lead to the secretion of IFN-γ (5). This is supported by the findings presented

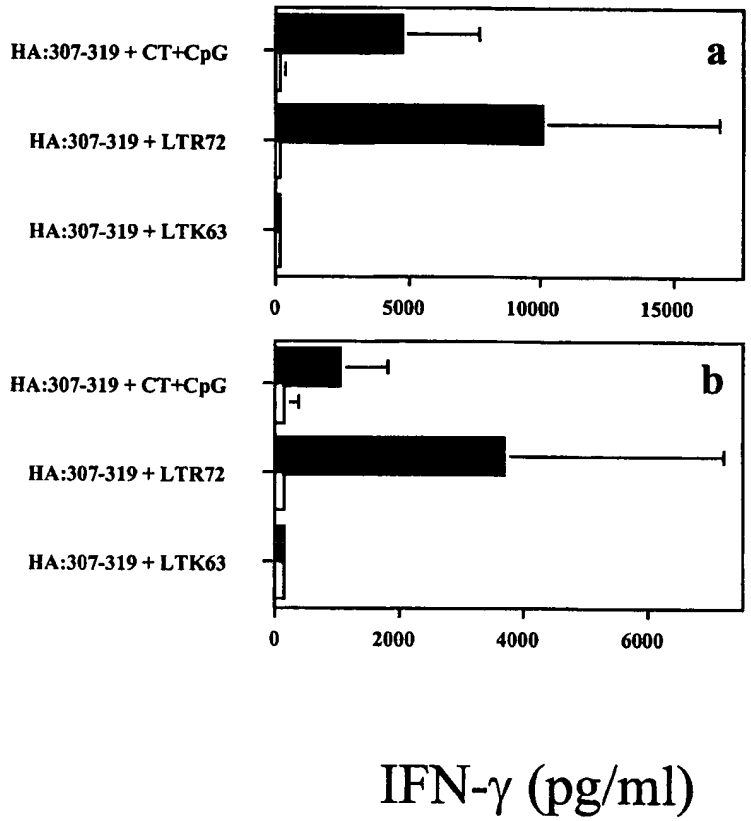

FIG. 6. Secretion of IFN-γ by HA:307-319 peptide-specific immune splenocytes after in vitro restimulation with 5 μg of homologous peptide (a) or 3×10^3 PFU of heat-inactivated influenza virus (b). Mice were coimmunized onto bare skin with 100 μg of HA:307-319 peptide plus 50 μg of LTK63, with 50 μg of LTR72, or with a mixture of 100 μg of CpG-ODN 1668 and 50 μg of CT. After 72 h of culture, the supernatants were collected and assayed for IFN-γ by ELISA. Results are also shown for medium alone (□).

here demonstrating different IFN-γ secretion profiles elicited by LT and its mutants LTR72 and LTK63 (Fig. 4).

Taken together, these findings lead us to conclude that adjuvants such as the LTK63 and LTR72 mutants, which have no or low residual toxicity, hold much promise for the future application of vaccines onto bare skin of humans.

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